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<b>(54) Title:</b> MONOCLONAL ANTIBODIES TO PUTATIVE HCV ENVELOPE REGION AND METHODS FOR USING SAME  <b>(57) Abstract</b>  Monoclonal antibodies which specifically bind to putative Hepatitis C Virus (HCV) envelope region. Also provided are hybridoma cell lines which secrete these monoclonal antibodies, methods for using these monoclonal antibodies, and assay kits which contain these monoclonal antibodies.		

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5 MONOCLONAL ANTIBODIES TO PUTATIVE HCV ENVELOPE REGION  
AND METHODS FOR USING SAME

Background of the Invention

This invention relates generally to antibodies which specifically bind to hepatitis C virus (HCV), and more specifically, relates to a panel of novel hybridoma cells lines which secrete  
10 monoclonal antibodies to putative HCV envelope regions, and methods for using these monoclonal antibodies.

Descriptions of hepatitis diseases causing jaundice and icterus have been known to man since antiquity. Viral hepatitis is now known to include a group of viral agents with  
15 distinctive viral organization protein structure and mode of replication, causing hepatitis with different degrees of severity of hepatic damage through different routes of transmission. Acute viral hepatitis is clinically diagnosed by well-defined patient symptoms including jaundice, hepatic tenderness and an elevated level of liver transaminases such as aspartate transaminase and alanine transaminase.

20 Serological assays currently are employed to further distinguish between hepatitis-A and hepatitis-B. Non-A non-B Hepatitis (NANBH) is a term first used in 1975 that described cases of post-transfusion hepatitis not caused by either hepatitis A virus or hepatitis B virus. Feinstone et al., New Engl. J. Med. 292:454-457 (1975). The diagnosis of NANBH has been  
25 made primarily by means of exclusion on the basis of serological analysis for the presence of hepatitis A and hepatitis B. NANBH is responsible for about 90% of the cases of post-transfusion hepatitis. Hollinger et al. in N. R. Rose et al., eds., Manual of Clinical Immunology, American Society for Microbiology, Washington, D. C., 558-572 (1986).

30 Attempts to identify the NANBH virus by virtue of genomic similarity to one of the known hepatitis viruses have failed thus far, suggesting that NANBH virus has a distinctive genomic organization and structure. Fowler et al., J. Med. Virol. 12:205-213 (1983), and Weiner et al., J. Med. Virol. 21:239-247 (1987). Progress in developing assays to detect antibodies specific for NANBH has been hampered by difficulties encountered in identifying  
35 antigens associated with the virus. Wards et al., U. S. Patent No. 4,870,076; Wards et al., Proc. Natl. Acad. Sci. 83:6608-6612 (1986); Ohori et al., J. Med. Virol. 12:161-178 (1983); Bradly et al., Proc. Natl. Acad. Sci. 84:6277-6281 (1987); Akatsuka et al., J. Med. Virol. 20:43-56 (1986).

In May of 1988, a collaborative effort of Chiron Corporation with the Centers for Disease Control resulted in the identification of a putative NANB agent, hepatitis C virus (HCV). M. Houghton et al. cloned and expressed in E. coli a NANB agent obtained from the infectious plasma of a chimp. Kuo et al., Science 244:359-361 (1989); Choo et al., Science 244:362-364 (1989). cDNA (copy DNA) sequences from HCV were identified which encode antigens that react immunologically with antibodies present in a majority of the patients clinically diagnosed with NANBH. Based on the information available and on the molecular structure of HCV, the genetic makeup of the virus consists of single stranded linear RNA (positive strand) of molecular weight approximately 9.5 kb, and possessing one continuous translational open reading frame. J. A. Cuthbert, Amer. J. Med. Sci. 299:346-355 (1990). It is a small enveloped virus resembling the Flaviviruses. Investigators have made attempts to identify the NANB agent by ultrastructural changes in hepatocytes in infected individuals. H. Gupta, Liver 8:111-115 (1988); D.W. Bradly J. Virol. Methods 10:307-319 (1985). Similar ultrastructural changes in hepatocytes as well as PCR amplified HCV RNA sequences have been detected in NANBH patients as well as in chimps experimentally infected with infectious HCV plasma. T. Shimizu et al., Proc. Natl. Acad. Sci. 87:6441-6444 (1990).

Considerable serological evidence has been found to implicate HCV as the etiological agent for post-transfusion NANBH. H. Alter et al., N. Eng. J. Med. 321:1494-1500 (1989); Estabien et al., The Lancet: Aug. 5:294-296 (1989); C. Van Der Poel et al., The Lancet Aug. 5:297-298 (1989); G. Sbolli, J. Med. Virol. 30:230-232 (1990); M. Makris et al., The Lancet 335:1117-1119 (1990). Although the detection of HCV antibodies eliminates 70 to 80% of NANBH infected blood from the blood supply system, the antibodies apparently are readily detected during the chronic state of the disease, while only 60% of the samples from the acute NANBH stage are HCV antibody positive. H. Alter et al., New Eng. J. Med. 321:1994-1500 (1989). The prolonged interval between exposure to HCV and antibody detection, and the lack of adequate information regarding the profile of immune response to various structural and non-structural proteins raises questions regarding the infectious state of the patient in the antibody negative phase during NANBH infection. Therefore, there is a need for the development of assay systems to identify acute infection to HCV and the presence of HCV.

#### Summary of the Invention

The present invention provides a panel of highly specific and novel monoclonal antibodies that can be employed for the detection of putative HCV envelope regions. The monoclonal antibodies specifically bind to peptides derived from the putative HCV envelope (ENV) gene. The hybridomas which secrete these monoclonal antibodies are identified as follows: Hybridoma cell line 16-407-209 (A.T.C.C. deposit No. HB 10601, secreting monoclonal antibody 16-407-209), and hybridoma cell line 16-803-174 (A.T.C.C. deposit No.

HB 10605, secreting monoclonal antibody 16-803-174). The specificity of these monoclonal antibodies enables the advantageous identification of putative HCV envelope region, which identification can be useful in differentiation studies as well as in the diagnosis and evaluation of HCV (NANB) infections.

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In a preferred assay format, a test sample which may contain HCV antigens is contacted with a solid phase to which a polyclonal or a monoclonal anti-HCV envelope region antibody or a fragment thereof has been bound, to form a mixture. This mixture is incubated for a time and under conditions sufficient for antigen/antibody complexes to form. The so-formed complexes then are contacted with an indicator reagent comprising a monoclonal or polyclonal antibody or a fragment thereof, specific for the HCV antigen which is attached to a signal generating compound, to form a second mixture. This second mixture is reacted for a time and under conditions sufficient to form antibody/antigen/antibody complexes. The presence of HCV antigen is determined by detecting the measurable signal generated. The amount of HCV present in the test sample, thus the amount of HCV antigen captured on the solid phase, is proportional to the amount of signal generated.

Alternatively, an indicator reagent comprising a monoclonal or polyclonal antibody, or fragment thereof, specific for HCV envelope region and a signal generating compound is added to a polyclonal or monoclonal anti-HCV antibody or fragment thereof coated on a solid phase and the test sample, to form a mixture. This mixture is incubated for a time and under conditions sufficient to form antibody/antigen/antibody complexes. The presence and amount of HCV present in the test sample, and thus the amount of HCV antigen captured on the solid phase, is determined by detecting the measurable signal. The amount of HCV present in the test sample is proportional to the amount of signal generated.

In another alternate assay format, one or a combination of more than one monoclonal antibody of the invention can be employed as a competitive probe for the detection of antibodies to putative HCV envelope region. For example, HCV envelope region proteins, either alone or in combination, can be coated on a solid phase. A test sample suspected of containing antibody to HCV envelope region then is incubated with an indicator reagent comprising a signal generating compound and a monoclonal antibody of the invention for a time and under conditions sufficient to form antigen/antibody complexes of either the test sample and indicator reagent to the solid phase or the indicator reagent to the solid phase. The reduction in binding of the monoclonal antibody to the solid phase can be quantitatively measured. A measurable reduction in the signal compared to the signal generated from a confirmed negative NANBH test sample would indicate the presence of anti-HCV envelope antibody in the test sample.

In yet another assay format, a test sample is contacted with a solid phase to which HCV proteins are attached and an indicator reagent comprising a monoclonal antibody or fragment thereof specific for HCV attached to a signal generating compound, to form a mixture. The mixture is incubated for a time and under conditions sufficient for antibody/antigen complexes to form. The presence of anti-HCV antibody present in the test sample is determined by detecting the measurable signal generated, and comparing the signal to the measured signal generated from a known negative sample. A measurable reduction of signal of the test sample, compared to the known negative sample's signal, is indicative of the presence of anti-HCV antibodies. Competitive assays for the detection of anti-HCV antibody using antigens free in solution also can be performed.

The presence of putative HCV envelope region can be detected in a tissue sample by contacting the tissue sample with an indicator reagent comprising a signal generating compound attached to a monoclonal antibody which specifically binds to HCV envelope region or fragment thereof, to form a mixture. This mixture is incubated for a time and under conditions sufficient for antigen/antibody complex to form. The presence of HCV envelope region present in the tissue sample is determined by detecting the signal generated.

Also provided are kits for using the monoclonal antibodies of the invention.

#### Brief Description of the Drawings

FIG. 1 is a map of the HCV GENOME representing the non-structural (NS) genes and the structural genes, core (C) and envelope (E).

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FIGS. 2 to 13 are photographs of Western blots showing the reactivity of the monoclonal antibodies of the invention, where

lanes 1 to 3 contain monoclonal antibodies against HCV 33C protein (6-296-534 in lane 1, 6-914-518 in lane 2 and 6-1070-110 in lane 3);

lanes 4-6 contain monoclonal antibodies against HCV CORE (13-975-157 in lane 4, 14-153-234 in lane 5 and 14-1350-210 in lane 6);

lanes 7 and 8 contain monoclonal antibodies against the putative HCV ENV region (16-407-209 in lane 7 and 16-803-174 in lane 8);

lanes 9-10 contain monoclonal antibodies against HCV C-100 (25-1518-105 in lane 9, 28-735-355 in lane 10);

line 11 contains monoclonal antibody against CKS (29-121-236 in lane 11);

lane 12 contains a normal mouse serum control; and

lane 13 contains a negative control of antibody diluent.

FIG. 2 is an electroblot of these monoclonal antibodies run against CKS-CORE;  
FIG. 3 is an electroblot of these monoclonal antibodies run against  $\lambda$ PL-CORE;  
FIG. 4 is an electroblot of these monoclonal antibodies run against  $\lambda$ PL-33C-CORE;  
FIG. 5 is an electroblot of these monoclonal antibodies run against CKS-33C;  
5 FIG. 6 is an electroblot of these monoclonal antibodies run against CKS-33C-BCD;  
FIG. 7 is an electroblot of these monoclonal antibodies run against CKS-BCD;  
FIG. 8 is an electroblot of these monoclonal antibodies run against CKS-B;  
FIG. 9 is an electroblot of these monoclonal antibodies run against CKS-E;  
FIG. 10 is an electroblot of these monoclonal antibodies run against CKS;  
10 FIG. 11 is an electroblot of these monoclonal antibodies run against SOD-100;  
FIG. 12 is an electroblot of these monoclonal antibodies run against CKS-A'BCD; and  
FIG. 13 is an electroblot of these monoclonal antibodies run against CKS-A"BCD.  
FIG. 14 is the amino acid sequence of the putative ENV domain of the HCV genome  
380-436.

15

#### Detailed Description of the Invention

The present invention provides novel monoclonal antibodies to putative HCV envelope region, methods for using the monoclonal antibodies, and kits which contain these monoclonal antibodies.

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The monoclonal antibodies of the present invention can be employed in various assay systems to determine the presence, if any, of putative HCV envelope region proteins in a test sample. Fragments of these monoclonal antibodies provided also may be used. For example, in a first assay format, a polyclonal or monoclonal anti-HCV envelope region antibody or  
25 fragment thereof, or a combination of these antibodies, which has been coated on a solid phase, is contacted with a test sample which may contain putative HCV envelope region proteins, to form a mixture. This mixture is incubated for a time and under conditions sufficient to form antigen/antibody complexes. Then, an indicator reagent comprising a monoclonal or a polyclonal antibody or a fragment thereof, which specifically binds to the putative HCV  
30 envelope region, or a combination of these antibodies, to which a signal generating compound has been attached, is contacted with the antigen/antibody complexes to form a second mixture. This second mixture then is incubated for a time and under conditions sufficient to form antibody/antigen/antibody complexes. The presence of putative HCV envelope region present in the test sample and captured on the solid phase, if any, is determined by detecting  
35 the measurable signal generated by the signal generating compound. The amount of putative HCV envelope region present in the test sample is proportional to the signal generated.

Alternatively, a polyclonal or monoclonal anti-HCV envelope region antibody or fragment thereof, or a combination of these antibodies which is bound to a solid support, the test sample and an indicator reagent comprising a monoclonal or polyclonal antibody or fragments thereof, which specifically binds to putative HCV envelope region, or a combination  
5 of these antibodies to which a signal generating compound is attached, are contacted to form a mixture. This mixture is incubated for a time and under conditions sufficient to form antibody/antigen/antibody complexes. The presence, if any, of putative HCV envelope region proteins present in the test sample and captured on the solid phase is determined by detecting the measurable signal generated by the signal generating compound. The amount of HCV  
10 proteins present in the test sample is proportional to the signal generated.

In another alternate assay format, one or a combination of one or more monoclonal antibodies of the invention can be employed as a competitive probe for the detection of antibodies to putative HCV envelope region. For example, putative HCV envelope region  
15 proteins, either alone or in combination, can be coated on a solid phase. A test sample suspected of containing antibody to putative HCV envelope region then is incubated with an indicator reagent comprising a signal generating compound and at least one monoclonal antibody of the invention for a time and under conditions sufficient to form antigen/antibody complexes of either the test sample and indicator reagent to the solid phase or the indicator  
20 reagent to the solid phase. The reduction in binding of the monoclonal antibody to the solid phase can be quantitatively measured. A measurable reduction in the signal compared to the signal generated from a confirmed negative NANBH test sample indicates the presence of anti-HCV envelope antibody in the test sample.

25 In yet another detection method, each of the monoclonal antibodies of the present invention can be employed in the detection of HCV antigens in fixed tissue sections, as well as fixed cells by immunohistochemical analysis.

In addition, these monoclonal antibodies can be bound to matrices similar to CNBr-  
30 activated Sepharose and used for the affinity purification of specific HCV proteins from cell cultures, or biological tissues such as blood and liver.

The monoclonal antibodies of the invention can also be used for the generation of chimeric antibodies for therapeutic use, or other similar applications.

35

The monoclonal antibodies or fragments thereof can be provided individually to detect putative HCV envelope region. Combinations of the monoclonal antibodies (and fragments thereof) provided herein also may be used together as components in a mixture or "cocktail" of



anti-HCV envelope region antibodies with antibodies to other HCV regions, each having different binding specificities. Thus, this cocktail can include both the monoclonal antibodies of the invention which are directed to putative HCV envelope region proteins and other monoclonal antibodies to other antigenic determinants of the HCV genome. Examples of other monoclonal antibodies useful for these contemplated cocktails include those to HCV C-100, HCV 33C and/or HCV CORE, disclosed in U. S. Serial No. 07/610,175 entitled MONOCLONAL ANTIBODIES TO HEPATITIS C VIRUS AND METHOD FOR USING SAME, and also those disclosed in Continuation-in-Part Applications of U.S.S.N. 07/610,175 entitled MONOCLONAL ANTIBODIES TO HCV CORE PROTEINS AND METHODS FOR USING SAME, U. S. Serial No. 648,473 and MONOCLONAL ANTIBODIES TO HCV 33C PROTEINS AND METHODS FOR USING SAME, U.S. Serial No. 648,477, which applications enjoy common ownership and are incorporated herein by reference

The polyclonal antibody or fragment thereof which can be used in the assay formats should specifically bind to putative HCV envelope region or other HCV proteins used in the assay, such as HCV C-100 protein, HCV 33C protein or HCV CORE protein. The polyclonal antibody used preferably is of mammalian origin; human, goat, rabbit or sheep anti-HCV polyclonal antibody can be used. Most preferably, the polyclonal antibody is rabbit polyclonal anti-HCV antibody. The polyclonal antibodies used in the assays can be used either alone or as a cocktail of polyclonal antibodies. Since the cocktails used in the assay formats are comprised of either monoclonal antibodies or polyclonal antibodies having different HCV specificity, they would be useful for diagnosis, evaluation and possibly for the prognosis of HCV infection, as well as for studying HCV protein differentiation and specificity.

Test samples which can be tested by the methods of the present invention described herein include human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, urine, biological fluids such as cell culture supernatants, fixed tissue specimens and fixed cell specimens. Solid supports are known to those in the art and include the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, and others.

The indicator reagent comprises a signal generating compound (label) which is capable of generating a measurable signal detectable by external means conjugated (attached) to a specific binding member for HCV. "Specific binding member" as used herein means a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. In addition to being an antibody member of a specific binding pair for HCV, the indicator reagent also can be a member of any specific binding pair, including either hapten-anti-hapten systems such as biotin or anti-

biotin, avidin or biotin, a carbohydrate or a lectin, a complementary nucleotide sequence, an effector or a receptor molecule, an enzyme cofactor and an enzyme, an enzyme inhibitor or an enzyme, and the like. An immunoreactive specific binding member can be an antibody, an antigen, or an antibody/antigen complex that is capable of binding either to HCV as in a sandwich assay, to the capture reagent as in a competitive assay, or to the ancillary specific binding member as in an indirect assay.

The various signal generating compounds (labels) contemplated include chromogens, catalysts such as enzymes, luminescent compounds such as fluorescein and rhodamine, chemiluminescent compounds, radioactive elements, and direct visual labels. Examples of enzymes include alkaline phosphatase, horseradish peroxidase, beta-galactosidase, and the like. The selection of a particular label is not critical, but it will be capable of producing a signal either by itself or in conjunction with one or more additional substances.

It is contemplated that the reagent employed for the assay can be provided in the form of a kit with one or more containers such as vials or bottles, with each container containing a separate reagent such as a monoclonal antibody, or a cocktail of monoclonal antibodies, employed in the assay.

## Materials and Methods

### Production of recombinant HCV antigens and immunogens

Synthetic peptides corresponding to regions within the putative ENV domain of the HCV genome were made by automated peptide synthesizer. The following peptides were constructed utilizing standard methods known in the art:

ENV 380-436  
405-436.

These peptides are described in pending United States patent application Serial No. 07/610,180 entitled HEPATITIS C ASSAY, which enjoys common ownership and which is incorporated herein by reference. FIG. 1 is a map of the HCV genome and the approximate locations of HCV regions. The amino acid sequence for the putative ENV domain of the HCV genome (p380-436) is shown in FIG. 14.

### Immunization of Mice

BALB/c mice (Charles River Laboratories, Charles River, NY), 6-8 weeks old, were initially immunized subcutaneously and intraperitoneally with 50 µg of the HCV peptide to the putative envelope region p380-436, in 100 µl of Freund's complete adjuvant (Difco, Detroit, MI). On day 15, 50 µg of the immunogen was diluted into 100 µl of phosphate buffered saline

(PBS), pH 7.2, and injected intravenously into the tail vein (J. Goding, Monoclonal Antibodies: Principles and Practice (New York; Academic Press, 1986)). Sera titers were not evaluated.

### Fusion

5           On day 18, mice were sacrificed and splenocytes were fused in a 1:1 ratio with the SP2/0 myeloma line according to known conventional methods [G. Kohler and C. Milstein, Nature (1975) 256:495-497; J. Goding, supra]. The cell fusion pellet was dispersed with 1 ml 50% polyethylene glycol (PEG) (American Type Culture Collection, MW 1450) and centrifuged in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Grand Island, NY). The cells were  
10           resuspended in HAT (hypoxanthine-aminopterin-thymidine)-selective IMDM with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) and plated at  $3 \times 10^5$  cells per 96-well tissue culture plates. Growth promoters included in the HAT media were 0.5% STM (RIBI Immunochem Research, Inc., Hamilton, MT) and 1% Origen Hybridoma Cloning Factor (Igen, Rockville, MD). Growth medium was replaced in culture wells post-fusion on day 5 and 7 using  
15           HT (hypoxanthine-thymidine) supplemented IMDM with 10% FBS.

### Enzyme Immunoassay (EIA)

          Culture supernates were EIA screened 10 days post-fusion against the immunizing antigen to detect hybrids secreting HCV specific antibody and a non-specific protein to  
20           eliminate any false positives (Langone & Van Vunakis, eds., Methods in Enzymology, 92:168-174, Academic Press [1983]). Polystyrene 96-well microtiter plates were coated overnight at room temperature with 50  $\mu$ l per well of a 1  $\mu$ g/ml of HCV peptide a.a. 380-436 in PBS. Any remaining binding sites on the polystyrene wells were blocked with 3% bovine serum albumin (BSA) (Intergen, Purchase, NY) in PBS for 30 minutes at room temperature. Plates were  
25           washed three times with distilled water. Fifty microliters of hybridoma tissue culture supernatants were incubated for 1 hour at room temperature in the wells, and the wells were washed three times with distilled water. Antibody binding to antigen was detected using goat anti-mouse IgG+M-horseradish peroxidase (HRPO) (Kirkegaard-Perry Laboratories [KPL], Gaithersburg, MD) diluted at a concentration of 1:1000 in the block solution and incubated 30  
30           minutes at room temperature. The plates were washed with distilled water and o-phenylenediamine substrate (OPD; Abbott Laboratories, Abbott Park, IL) was used as the chromogen. Plates were read at 492 nm. Hybrid cultures were regarded as potential HCV antibody-positive when the optical density (OD) was 3 times the negative control (NC) and significant preferential to the HCV antigen plate was observed compared to antibody binding of  
35           the irrelevant antigen coated plate, ie:  $>0.2$  OD difference and  $<0.2$  OD signal on the latter.

### Western Blot

Hybrid antibody specificity was confirmed with Western blot analysis (Towbin & Gordon, J. Immunol. Methods, 72:313-340 [1984]). HCV recombinant proteins and irrelevant proteins were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose, according to the manufacturer's instructions (Schleicher & Schuell, Keene, NH; Bio-Rad, Richmond, CA). The nitrocellulose strips were blocked with 1% bovine hemoglobin (Sigma Chemical Co., St. Louis, MO) and 0.5% Tween-20 (Fisher Scientific, Pittsburgh, PA) in PBS for 30 minutes at room temperature, then the strips were incubated with hybrid tissue culture supernatant. The strips were then washed in PBS and goat anti-mouse IgG+M-HRPO (KPL) added for 30 minutes. Antibody binding to the HCV antigen was visualized with 4-chloro-1-naphthol (Sigma) as the chromogenic substrate. Hybrid cultures were cloned and placed in cryostorage if HCV antibody specificity was demonstrated.

### Establishment of Clones

HCV specific hybrids were cloned by limiting dilution (Goding, Monoclonal Antibodies: Principles and Practices, 2nd ed, Academic Press, New York [1986]). Modifications included plating of the cultures in log<sub>10</sub> dilution series and selecting positive clones for expansion from plates which exhibited <20% growth per 96 well tissue culture plate. Culture supernates were tested after 10 days using the EIA and Western blot procedures described above. The selected clones were expanded for further evaluation and cryostored in 80% IMDM with 10% FBS (Hyclone) and 10% DMSO (Sigma).

### Monoclonal Antibody Isotype

Monoclonal antibody isotype was determined with the SBA Clonotyping System III kit (Southern Biotechnology Associates, Inc., Birmingham, AL) with modifications. EIA 96-well microtiter plates were coated overnight at room temperature with 100 µl/well of a 1:1000 dilution of goat anti-mouse IgG+M (H+L) (KPL). Plates were blocked for 30 minutes with 3% BSA in PBS and washed with water. Culture samples were added to the wells, incubated for 1 hour, and washed with water. The kit's goat anti-mouse subtype specific conjugates were added for a 30 minute incubation period. Following a water wash, color was identified with OPD substrate. The goat anti-mouse isotype specific conjugate that bound to the mouse immunoglobulin and displayed a >0.1 OD at 492 nm signaled the subtype.

### Monoclonal Antibody Production

Clones selected for further evaluation were scaled up in tissue culture T-flasks and 10<sup>6</sup> cells were injected into the peritoneal cavity of pre-pristaned BALB/c mice (Charles River Biotechnical Services, Inc., Wilmington, MA) (see Hurrell, supra). The resulting ascites fluid was harvested 7-10 days after injection, centrifuged, and stored at -20°C. The IgG antibody was

affinity purified on Protein A (Pharmacia-LKB Biotechnologies, Piscataway, NJ) utilizing the automated OROS purification system Model 100 (see Goding, supra, for basic principles). The IgM antibodies were purified by molecular sizing on a S-300 column (Pharmacia-LKB).

- 5 All the following characterization information was performed with purified monoclonal antibody.

#### Isoelectric Focusing (IEF)

A cell line quality control to ensure consistency of frozen lots included measuring the antibody pI point on an IEF gel apparatus (Bio-Rad) which separates proteins based on net  
10 charge. Briefly, a bis-acrylamide-riboflavin solution was applied to an acrylamide gel, exposed to fluorescent lighting for 1 hour, then stored overnight at 4°C. A 1 µg sample of monoclonal antibody and standards were overlayed on the gel and electrophoresed over a 1-2 hour period. Following a series of fixatives and washes, the gel was silver stained (Bio-Rad). The pI value of the monoclonal antibody was calculated by migratory distance through the gel and was directly  
15 compared to the protein standards' migratory distance of known pI values. The distinctive finger print banding pattern reflected the pI microheterogeneity between independently produced lots of antibody (Hamilton, R.G., Reimer, C.B., Rodkey, L.S. (1987) Quality control of murine monoclonal antibodies using isoelectric focusing affinity immunoblot analysis. Hybridoma 6:205-217).

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#### EIA and Western Blot Specificity of Monoclonal Antibodies

All monoclonal antibodies noted herein were screened on an assortment of available recombinant HCV antigens as disclosed in U. S. Patent Application Serial No. 07/572,822 entitled HEPATITIS C ASSAY UTILIZING RECOMBINANT PROTEINS, which enjoys common  
25 ownership and is incorporated herein by reference. The procedures were as outlined above. The multiple antigen screening technique confirmed the HCV specificity and excluded the HCV non-specific CKS, λPL, or linker-arm reactivity of the monoclonal antibodies.

#### EIA Epitope Competition Studies

30 To investigate specificity and antigen binding distinctions, epitope grouping experiments were performed utilizing biotin labeled and unlabeled monoclonal antibodies (Langone & Van Vunakis, Methods in Enzymology, 92:242-253, Academic Press [1983]). Briefly, the antibodies were labeled with NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Microtiter wells were coated with the immunogen  
35 as previously described. First, log<sub>2</sub> dilutions of the unlabeled antibody were pre-incubated in the wells for 15 minutes, followed by the addition of a fixed amount of biotinylated antibody (the dilution in a direct EIA of the biotinylated antibody alone which gave a value of 50% of the maximum absorbance value) and incubated for 20 minutes. Plates were washed three times

with water. Diluted streptavidin-HRPO (Zymed, South San Francisco, CA) was added to the wells and incubated for 30 minutes. The plates were washed again and OPD color developed as previously described. The absorbance was read at 492 nm. Antibodies of the same or related epitope had signal blocked or inhibited by >50%. No inhibition was observed with antibodies of distinct specificity. This was performed reciprocally for antibodies produced with the ENV regions.

#### Peptide Inhibition Assays

Synthetic peptides were synthesized as previously described for HCV amino acid sequences 385-436 and 405-436. These peptides were employed in competition assays according to the procedure previously described by substituting serial dilutions of the peptides in place of the unlabeled antibody. Fifty microliters of labeled antibody (50% maximum absorbance value) was pre-incubated with the peptides for 15 minutes in a separate 96-well tissue culture dish. Next, 50  $\mu$ l of the peptide and labeled monoclonal antibody mixture was added to the previously blocked antigen coated EIA plate and incubated for 20 minutes. Streptavidin-HRPO goat anti-mouse conjugated (Zymed) was employed to detect the immune complexes formed.

#### RIA Reciprocal Competition

Beads coated with the appropriate antigen or peptide were incubated with 100  $\mu$ l of unlabeled monoclonal antibody diluted into recalcified negative human plasma (NHP, testing negative for anti-HCV, anti-HIV and HBsAg) at monoclonal antibody concentrations of 1-20  $\mu$ g/ml. 100  $\mu$ l of radiolabeled antibody at 1 to 4  $\mu$ Ci/ml diluted into HTLV I kit specimen diluent (containing detergent, animal sera, buffer, available from Abbott Laboratories, Abbott Park, IL) was incubated with the bead for 2 hours at 45 $^{\circ}$  or 18-20 hours at 20-25 $^{\circ}$ C. Beads were washed and counted for radioactivity.

#### HCV Antigen Assays

Beads coated with one or a cocktail of anti-HCV monoclonal antibodies were incubated with 200  $\mu$ l of specimen for 2 hours at 40-45 $^{\circ}$ C or 18-20 hours at 20-25 $^{\circ}$ C. Beads were washed with distilled water and then incubated with 200  $\mu$ l of radiolabeled anti-HCV monoclonal antibody (one or more) for 2 hours at 45 $^{\circ}$ C. Beads were washed and counted in a gamma counter.

#### Characterization of monoclonal antibody

Monoclonal antibodies against the HCV ENV domain (380-436) AND (405-436) are characterized in Tables 1 and 2. Referring to FIGS. 2 to 13, the reactivities summarized below in Table 1 are shown in lanes 7 and 8. Lanes 1 to 3 contains monoclonal antibodies against HCV

33C protein (6-296-534 in lane 1, 6-914-518 in lane 2 and 6-1070-110 in lane 3); lanes 4-6 contain monoclonal antibodies against HCV CORE (13-975-157 in lane 4, 14-153-234 in lane 5 and 14-1350-210 in lane 6); lanes 7 and 8 contain monoclonal antibodies against the putative HCV ENV region (16-407-209 in lane 7 and 16-803-174 in lane 8); lanes 9-11 contain monoclonal antibodies against HCV C-100 (25-1518-105 in lane 9, 28-735-355 in lane 10; CKS control monoclonal antibody 29-121-236 in lane 11); lane 12 contains a normal mouse serum control; and lane 13 contains a negative control.

TABLE 1

10

## Reactivity on Western Blot

Cell Line	CKS- core	$\lambda$ PL- core	$\lambda$ PL- c33- core	CKS- c33	CKS- c33- BCD	CKS- BCD	CKS- B	CKS- E	CKS- SOD 100	CKS- ABCD	CKS- A*BCD
16-407-209	-	-	-	-	-	-	-	-	-	-	-
16-803-174	-	-	-	-	-	-	-	-	-	-	-

15

20

TABLE 2.

## Reactivity on EIA

Group #	Cell Line	pl	Isotype	ENV 380-436	ENV 405-436	$\lambda$ PL 33c- core	CKS- 33c
1	16-407-209	7.0	IgG3k	+	+	-	-
2	16-803-174		IgMk	+	+	-	-

25

The following examples demonstrate the advantages and utility of this invention for serodiagnosis of HCV by describing methods for the clinical utility of these monoclonal antibodies. These examples are meant to illustrate, but not to limit, the spirit and scope of the invention.

30

## EXAMPLES

35

## Example 1

Anti-HCV ENV Competitive Assay

Ten (10) specimens from blood bank donors with elevated alanine aminotransferase (ALT) were tested in a competitive one-step assay described hereinabove for "RIA Reciprocal Competition" for the detection of anti-HCV ENV. The data are presented in Table 3. Referring to Table 3, if >25% inhibition is considered reactive in this assay, then one of ten human test samples with elevated ALT was reactive for anti-HCV ENV.

40

TABLE 3

## Anti-ENV Immunoassay

5		Bead: 380 peptide (p380-436)									
10	Specimen	Label: 16-958					Label 16-407				
		CPM	AVG	S/N	%Inhib	Result	CPM	AVG	S/N	%Inhib	Result
10	NC	1535	1621				1032	1015			
		1779					977				
		1550					1036				
15	27	2862	2571	1.59	-58.605799	-	1079	1136	1.12	-11.8719	-
		2280					1192				
15	238	1295	1295	0.75	24.9228871	+	767	798	0.79	21.42857	+
		1139					828				
20	135	1606	2054	1.27	-26.711906	-	869	852	0.84	16.10837	-
		2502					834				
20	163	3128	3058	1.89	-88.648982	-	1377	1464	1.44	-44.2365	-
		2988					1551				
25	173	2240	2154	1.33	-32.850093	-	1128	1034	1.02	-1.82266	-
		2067					939				
25	220	3503	3232	1.99	-99.352252	-	1119	1065	1.05	-4.87685	-
		2960					1010				
25	252	4208	4149	2.56	-155.92227	-	1222	1150	1.13	-13.5005	-
		4098					1078				
30	283	3330	3197	1.97	-97.193091	-	1059	1039	1.02	-2.31527	-
		3063					1018				
30	28	4014	3829	2.36	-136.21221	-	1215	1306	1.29	-28.67	-
		3644					1397				
35	290	3701	3542	2.19	-118.50709	-	1314	1290	1.27	-27.0443	-
		3883					1265				
35	16-803-174	41	46	0.03	97.1622455	+	220	259	0.26	74.48276	+
		51					298				
35	PC813	1858	1876	1.16	-15.73103	-	1065	1138	1.12	-12.1511	-
		1878					1088				
		1892					1262				

## Example 2

HCV ENV Antigen Assay

40

Antigen assays were conducted using the HCV Antigen Assay described herein as "HCV Antigen Assays." Results from a two step ENV antigen assay are shown in Table 4. The most sensitive assay for detection of 380-436 ENV synthetic peptide was a bead coated with monoclonal antibodies 16-407-209 and 16-803-174 label. The sensitivity for synthetic peptide was less than 10 µg/ml.

45



TABLE 4  
HCV ENV Antigen Assay

5	Specimen	(16-407-209 Bead: 16-803-174 Label)				(16-803-174 Bead: 16-407-209 Label)			
		CPM	AVG	S/N	Result	CPM	AVG	S/N	Result
	NC	205 213 199	206			230 235 198	221		
10	NC + 20%NaAc	228	229 229	1.11	-	256	256 256	1.16	-
	380-436 ) (20 µg/ml	1537 1402	1470	7.13	+	746 672	709	3.21	+
15	380-436 (200 µg/ml)	2685 2464	2575	12.50	+	646 673	660	2.98	+

### Example 3

#### HCV Antibody Test Employing Cocktails of Monoclonal Antibodies

20       Antigen assays were conducted using the HCV Antigen Assay described herein as "HCV Antigen Assays." Another variation of this assay used a cocktail of monoclonal antibody on the bead (16-407, 16-803, and 16-1291) and a cocktail label (16-407 and 16-803). It was found that one specimen of 36 obtained from the Interstate Blood Bank (designated as number 13) showed significant reactivity in this assay.

25

      Thus, the novel monoclonal antibodies of the invention can be used in a variety of ways. These monoclonal antibodies can be used for immunoprecipitation of amplified product and detection of HCV nucleic acid microparticles or carrier coated with anti-HCV monoclonal antibody used to capture virus or viral protein associated with HCV RNA. Then detection methodology for RNA may be used. An example of this type of assay is taught in pending U. S. patent application Serial No. 07/568,663, entitled A METHOD FOR AMPLIFYING AND DETECTING A TARGET NUCLEIC ACID SEQUENCE, which enjoys common ownership and is incorporated herein by reference.

35

      These monoclonal antibodies also can be used for localization of HCV antigens within the cell using HCV monoclonal antibody tagged directly (fluorescence, colloidal gold, etc.) or using secondary tagged anti-mouse antibody. Histopathology of disease may be tracked. Further, the detection of native or recombinant HCV antigens in sera, tissue, cells, culture media, or body fluid using individual monoclonal antibodies in a sandwich configuration or a cocktail of monoclonal antibodies on the solid phase and in the detection system.

40

      One step antigen assays using monoclonal antibodies against non overlapping epitopes may also be performed. Some monoclonal antibodies may recognize antigenic

epitopes not recognized by the infected individual and therefore may be possible to recognize serum Ag both free and bound with human antibody. Furthermore, "cryptic" or hidden antigens or antigenic determinants may be uncovered by treatment of specimen with detergent or reducing agent or both. For example, CORE antigen may exist in a capsid form  
5 covered by the virus envelope. Stripping the envelope with detergent should expose CORE antigen. Monoclonal antibodies may also offer pragmatic advantages over high titer polyclonal antibody in giving greater sensitivity in assay or allowing shorter incubation times.

Further, antibody immunoassays, one or two step competitive assays, were developed  
10 in which anti-HCV competed with labeled anti-HCV monoclonal antibody for binding to a limited number of antigenic sites. A more sensitive competitive assay may be developed in which human anti-HCV binds to HCV Ag in solution blocking or inhibiting the HCV Ag binding in HCV Ag sandwich assay. Competitive assays using monoclonal antibodies allow a more precise mapping of human antibody epitopes and may be useful for determining virus neutralizing  
15 antibody epitopes. Some monoclonal antibodies may have virus neutralizing activity. Finally, monoclonal antibodies should be useful in immunoaffinity purification of native viral and recombinant HCV antigens and proteins.

The hybridoma cell lines which secrete the monoclonal antibodies of the invention are  
20 identified as hybridoma cell line 16-407-209 (secreting monoclonal antibody 16-407-209) and hybridoma cell line 16-803-174 (secreting hybridoma cell line 16-803-174). These hybridoma cell lines were deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on November 16, 1990 and were accorded the following deposit numbers: Hybridoma cell line 16-407-209 was accorded A.T.C.C. deposit No. HB 10601, and  
25 hybridoma cell line 16-803-174 was accorded A.T.C.C. deposit No. HB 10605.

Other variations of applications of the use of the unique monoclonal antibodies provided herein include the detection of HCV antigen in immune complexes, or latent and/or cryptic antigens, and/or associated with viral nucleic acid for detection of the nucleic acid by  
30 PCR, LCR, or by direct hybridization. Still other variations and modifications of the specific embodiments of the invention as set forth herein will be apparent to those skilled in the art. Accordingly, the invention is intended to be limited only in accordance with the appended claims.

## WHAT IS CLAIMED IS:

1. A monoclonal antibody which specifically binds to putative HCV envelope region.  
5
2. The monoclonal antibody of claim 1 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by hybridoma cell line HB 10601.
3. The monoclonal antibody of claim 1 wherein said monoclonal antibody has the  
10 binding specificity of the monoclonal antibody secreted by hybridoma cell line HB 10605.
4. A monoclonal antibody secreted by A.T.C.C. deposit No. HB 10601.
5. A monoclonal antibody secreted by A.T.C.C. deposit No. HB 10605.  
15
6. A hybridoma cell line which secretes a monoclonal antibody which specifically binds to putative HCV envelope region.
7. The hybridoma cell line of claim 6 having the identifying characteristics of  
20 hybridoma cell line A.T.C.C. deposit No. HB 10601.
8. The hybridoma cell line of claim 6 having the identifying characteristics of hybridoma cell line A.T.C.C. deposit No. HB 10605.
9. A hybridoma cell line A.T.C.C. deposit No. HB 10601.  
25
10. A hybridoma cell line A.T.C.C. deposit No. HB 10605.
11. A method for determining the presence of HCV in a test sample which may  
30 contain HCV, comprising:
  - a. contacting the test sample with at least an anti-HCV envelope region antibody attached to a solid phase which antibody specifically binds to HCV envelope region, to form a mixture;
  - b. incubating said mixture for a time and under conditions sufficient to  
35 form antigen/antibody complexes;
  - c. contacting said complexes with an indicator reagent comprising a signal generating compound capable of generating a measurable detectable signal attached to an anti-HCV envelope region antibody, to form a second mixture;

d. incubating said second mixture for a time and under conditions sufficient to form antibody/antigen/antibody complexes; and

e. determining the presence of HCV in the test sample by detecting the measurable signal generated.

5

12. The method of claim 11 wherein the amount of HCV present in the test sample is proportional to said measurable signal.

13. The method of claim 12 wherein the signal generating compound is selected from the group consisting of a luminescent compound, a chemiluminescent compound, an enzyme and a radioactive element.

10

14. The method of claim 13 wherein said enzyme is selected from the group consisting of horseradish peroxidase, alkaline phosphatase and beta-galactosidase.

15

15. The method of claim 14 wherein said enzyme is horseradish peroxidase.

16. The method of claim 11 wherein the anti-HCV antibody attached to the solid phase is a polyclonal antibody.

20

17. The method of claim 11 wherein said anti-HCV envelope region antibody attached to the solid phase is a monoclonal antibody.

18. The method of claim 17 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the hybridoma A.T.C.C. deposit No. HB 10601.

25

19. The method of claim 17 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the hybridoma A.T.C.C. deposit No. HB 10605.

30

20. The method of claim 11 wherein said indicator reagent comprises a signal generating compound attached to a polyclonal antibody.

35

21. The method of claim 11 wherein said indicator reagent comprises a signal generating compound attached to a monoclonal antibody.

22. The method of claim 21 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the hybridoma cell line A.T.C.C. deposit No. HB 10601.

5 23. The method of claim 21 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the hybridoma cell line A.T.C.C. deposit No. HB 10605.

24. A method for determining the presence and amount of HCV which may be present in a test sample, comprising:

- 10 a. contacting a test sample with a polyclonal or monoclonal anti-HCV envelope region antibody attached to a solid phase and an indicator reagent comprising a monoclonal or polyclonal antibody which specifically binds to HCV envelope region attached to a signal generating compound, to form a mixture;
- 15 b. incubating said mixture for a time and under conditions sufficient to form antibody/antigen/antibody complexes;
- c. determining the presence of HCV present in the test sample by detecting the measurable signal as an indication of the presence of HCV in the test sample.

20 25. The method of claim 24 wherein the amount of HCV present in the test sample is proportional to the measurable signal generated.

26. The method of claim 24 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the hybridoma cell line A.T.C.C. deposit No. HB 10601.

27. The method of claim 24 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the hybridoma cell line A.T.C.C. deposit No. HB 10605.

30 28. A competitive assay method for determining the presence and amount of HCV antibody which may be present in a test sample, comprising:

- 35 a. contacting a test sample suspected of containing HCV antibodies with a solid phase coated with HCV envelope proteins and an indicator reagent comprising a signal generating compound and a monoclonal antibody which specifically binds to HCV envelope proteins, for a time and under conditions sufficient to form antigen/antibody complexes of the test sample and solid phase and/or indicator reagent and solid phase;

b. determining the presence of HCV antibody present in the test sample by detecting the reduction in binding of the indicator reagent to the solid phase as compared to the signal generated from a negative test sample to indicate the presence of HCV antibody in the test sample

5

29. The method of claim 28 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the hybridoma cell line A.T.C.C. deposit No. HB 10601.

10

30. The method of claim 28 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the hybridoma cell line A.T.C.C. deposit No. HB 10605.

15

31. The method of claim 28 wherein the signal generating compound is selected from the group consisting of a luminescent compound, a chemiluminescent compound, an enzyme and a radioactive element.

20

32. The method of claim 31 wherein said enzyme is selected from the group consisting of horseradish peroxidase, alkaline phosphatase and beta-galactosidase.

33. The method of claim 32 wherein said enzyme is horseradish peroxidase.

34. An assay kit for determining the presence of HCV in a test sample comprising:  
a container containing at least one monoclonal antibody which specifically  
25 binds to putative HCV envelope region.

35. The assay kit of claim 34 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the cell line A.T.C.C. deposit No. HB 10601.

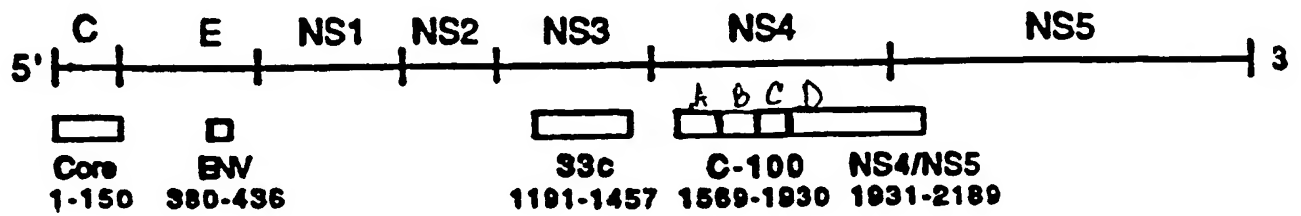
30

36. The assay kit of claim 34 wherein said monoclonal antibody has the binding specificity of the monoclonal antibody secreted by the cell line A.T.C.C. deposit No. HB 10605.

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**FIG. 1**

## HCV GENOME



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FIG. 2

CKS-CORE

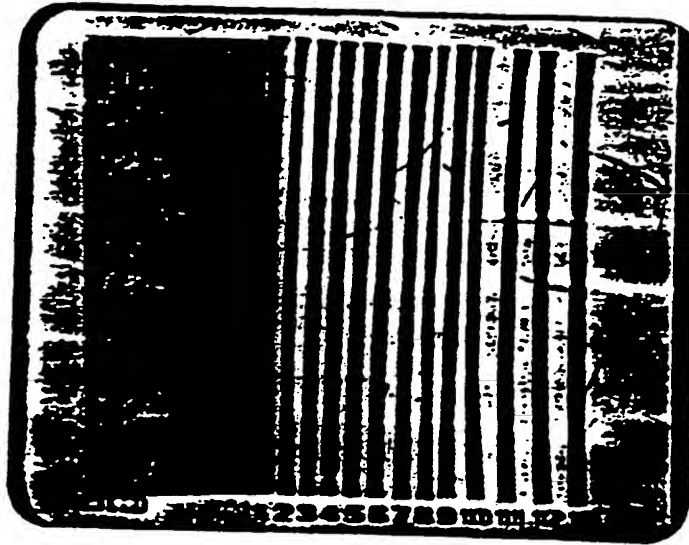
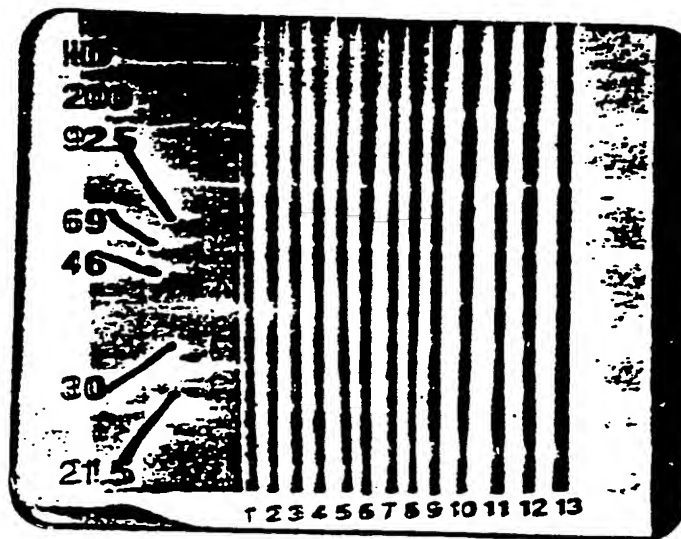


FIG. 3

APL-CORE





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FIG. 4

NPL-33-LORE

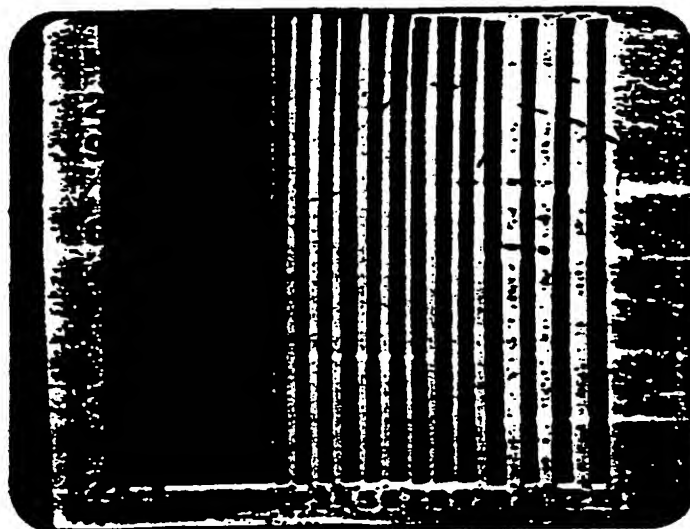
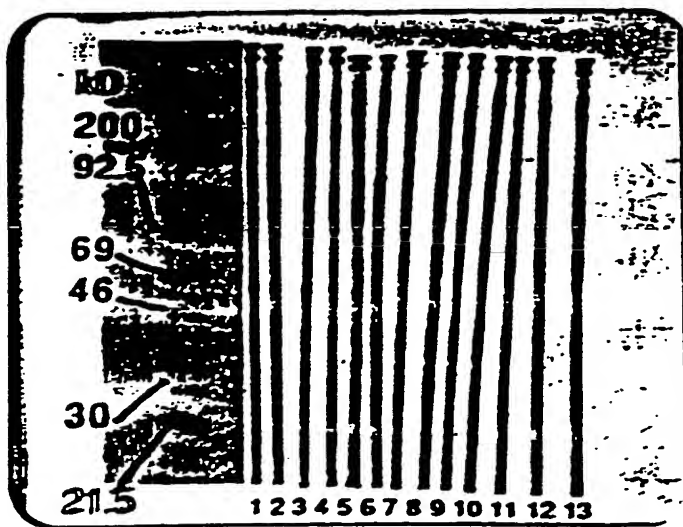


FIG. 5

CKS-33



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FIG. 6

CKS-33-BCD

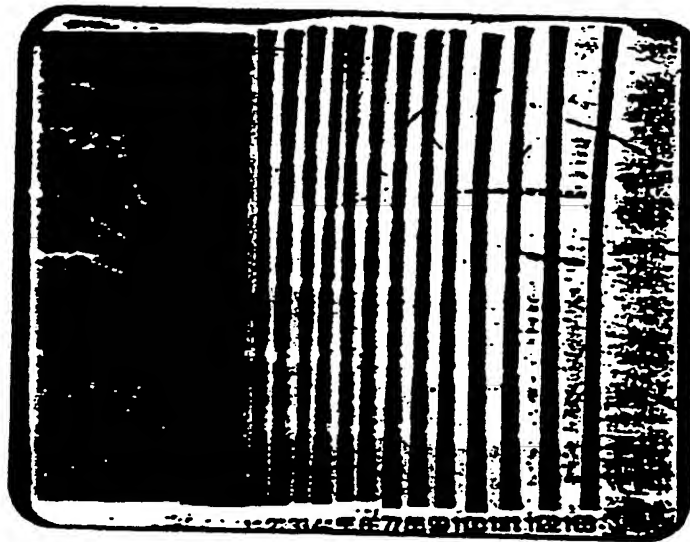
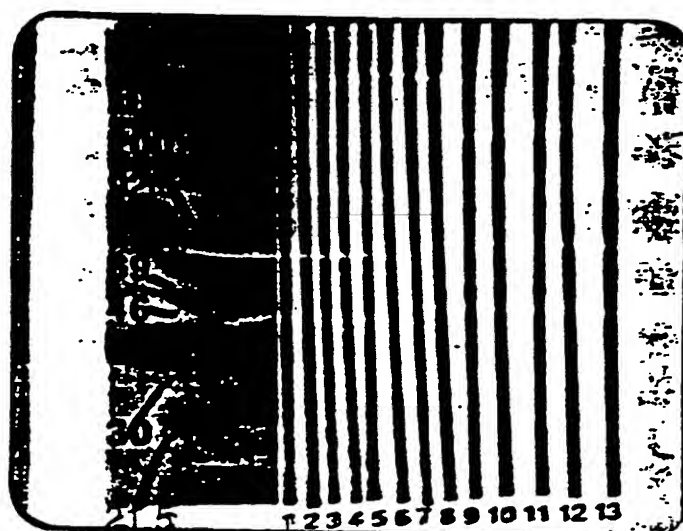


FIG. 7

CKS-BCD



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FIG. 8

CKS-B

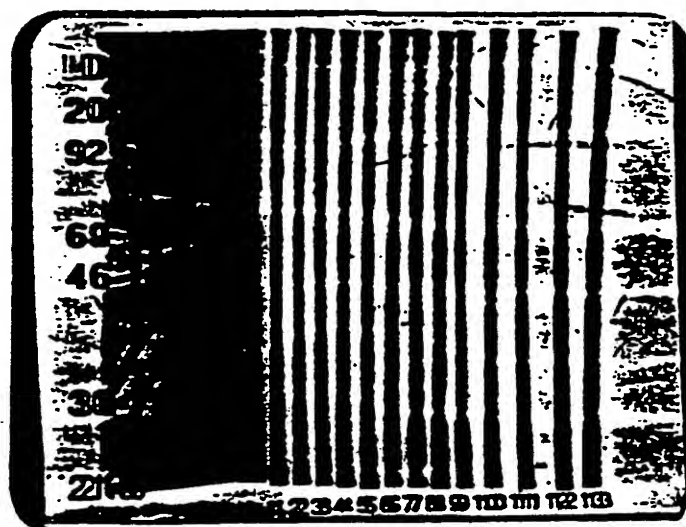
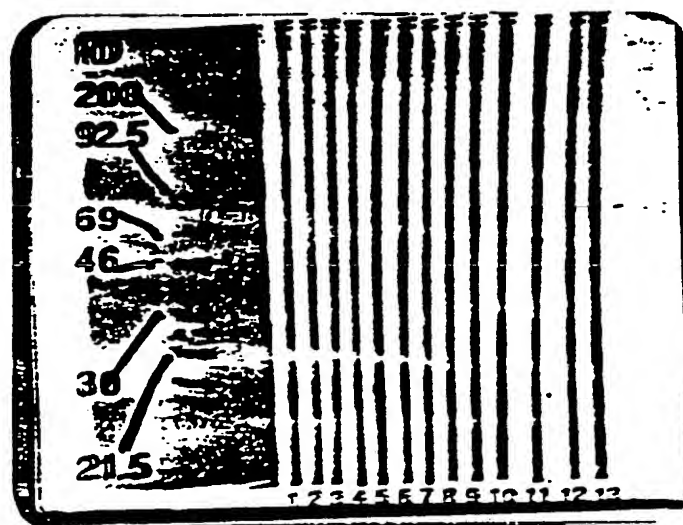


FIG. 9

CKS-E



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FIG. 10

CKS

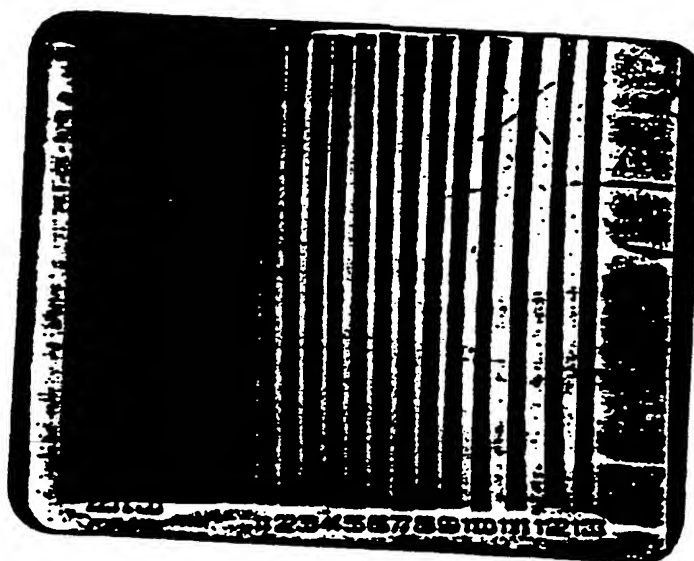
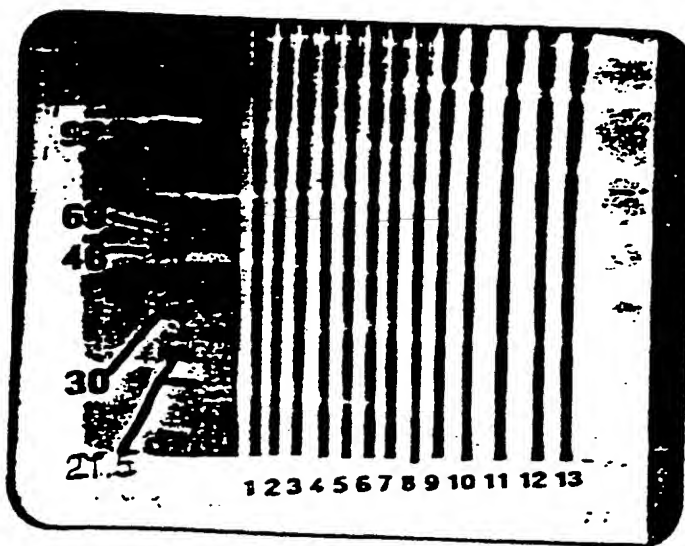


FIG. 11

SOD-100



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FIG. 12

CKS-A'BGD

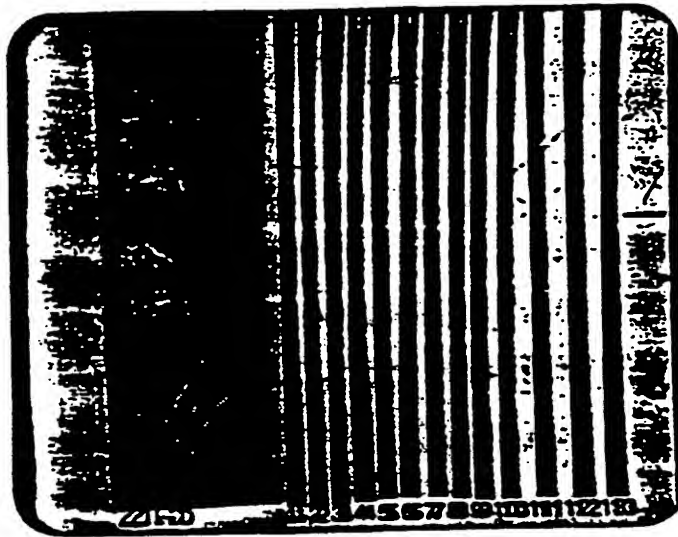
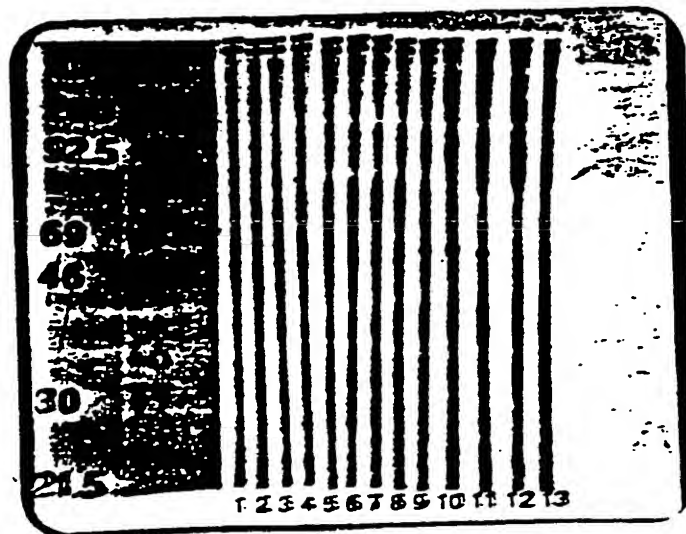


FIG. 13

CKS-A"BGD



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p380  
(380-436)

H -Gly-Val-Asp-Ala-Glu-Thr-His-Val-Thr-Glu-Gly-  
 10  
 Gly-Ser-Ala-Gly-His-Thr-Val-Ser-Gly-Phe-Val-Ser-  
 20  
 Leu-Leu-Ala-Pro-Gly-Ala-Lys-Gln-Asn-Val-Gln-  
 30  
 Leu-Ile-Asn-Thr-Asn-Gly-Ser-Trp-His-Leu-Asn-  
 40  
 Ser-Thr-Ala-Leu-Asn-Cys-Asn-Asp-Ser-Leu-Asn-  
 50  
 Thr-Gly- OH

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00687

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): C07K 15/28; C12P 21/08; C12N 5/00; C12Q 1/70 US CL : 530/388.3; 435/240.27, 5		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	530/388.3; 435/240.27, 5	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
APS, Dialog		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>8</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	EP, A, 0,318,216 (Houghton et al.) 31 May 1989. See the abstract and the claims.	1-36
Y	Japanese Journal of Experimental Medicine, Volume 60, No. 4, issued 1990, H. Okamoto et al., "Enzyme-Linked Immunosorbent Assay for Antibodies against the Capsid Protein of Hepatitis C Virus with a Synthetic Oligopeptide," pages 223-233, see the abstract.	1-36
Y	Japanese Journal of Cancer Research, Volume 81, issued November, 1990, N. Kato et al., "A Structural Protein Encoded by the 5' Region of the Hepatitis C Virus Genome Efficiently Detects Viral Infection," pages 1092-1094, see Figures 1 and 2 and page 1094, column 2.	1-36
Y	Nucleic Acids Research, Volume 18, No. 15, issued 1990, K. Takeuchi et al., "Nucleotide sequence of core and envelope genes of the hepatitis C virus genome derived directly from human healthy carriers," page 4626, see entire document.	1-36
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>15</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>		Date of Mailing of this International Search Report <sup>2</sup>
29 APRIL 1992		12 MAY 1992
International Searching Authority <sup>1</sup>		Signature of Authorized Officer <sup>20</sup>
ISA/US		<i>Deborah E. Finner</i> for Donna C. Wortman

